February 2012

EpiTect® Methyl II PCR Array Handbook

For pathway or disease-focused profiling of regional DNA methylation using MethylScreen™ technology

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Kit Contents

EpiTect Methyl II Signature PCR Arrays

Catalog no. 335212

Format	A	C	D	E	F	G
96-well plate containing lyophilized assays	2, 12, or 24	2, 12, or 24	2, 12, or 24	–	2, 12, or 24	–
384-well plate containing lyophilized assays	–	–	–	4	–	4
Optical thin-wall 8-cap strips (12 per plate)	24, 144, or 288	–	24, 144, or 288	–	–	–
Optical adhesive film (1 per plate)	–	2, 12, or 24	–	4	2, 12, or 24	4
384EZLoad Covers (set of 4 covers per plate)	–	–	–	4	–	4

EpiTect Methyl II Complete PCR Arrays

Catalog no. 335222

Format	A	C	D	E	F	G
96-well plate containing lyophilized assays	8	8	8	–	8	–
384-well plate containing lyophilized assays	–	–	–	2, 12, or 24	–	2, 12, or 24
Optical thin-wall 8-cap strips (12 per plate)	96	–	96		–	–
Optical adhesive film (1 per plate)	–	8	–	2, 12, or 24	8	2, 12, or 24
384EZLoad Covers (set of 4 covers per plate)	–	–	–	2, 12, or 24	–	2, 12, or 24

EpiTect Methyl II PCR Array specifications

Cat. no. and array format	Number of genes per plate	Plate format	Number of DNA samples per plate	µg gDNA per sample*	Number of DNA samples per kit
335212 A, C, D, F	22	96-well	1	1	12
335222 A, C, D, F	94	96-well	1	4	3
335222 E, G	94	384-well	1	2	6
335212 E, G	22	384-well	4	0.5	12

* Recommended amount for optimal PCR Array performance.

Array format and real-time cycler compatibility

Array format	Suitable real-time cyclers	Plate format
A	Applied Biosystems® 5700, 7000, 7300, 7500 Standard, 7700, 7900HT Standard; Bio-Rad® iCycler®, iQ™ 5, MyiQ™, MyiQ2™, Bio-Rad/MJ Research Chromo4™; Eppendorf® Mastercycler® ep realplex 2, 2s, 4, 4s; Stratagene® Mx3005P®, Mx3000P®	96-well
C	Applied Biosystems 7500 FAST, 7900HT FAST, StepOnePlus™	96-well
D	Bio-Rad CFX96™, Bio-Rad/MJ Research Opticon 2®; Stratagene Mx4000®	96-well
E	Applied Biosystems 7900HT (384-well block); Bio-Rad CFX384™	384-well
F	Roche® LightCycler® 480 (96-well block)	96-well
G	Roche LightCycler 480 (384-well block)	384-well

Storage

The EpiTect Methyl II Signature PCR Array and EpiTect Methyl II Complete PCR Array are shipped at ambient temperature, on ice, or on dry ice depending on the destination and accompanying products. Upon receipt, store at –20°C. If stored under these conditions, EpiTect Methyl II Signature PCR Arrays and EpiTect Methyl II Complete PCR Arrays are stable for 6 months after receipt.

Quality Control

In accordance with QIAGEN's Quality Management System, each lot of EpiTect Methyl II Signature PCR Array and EpiTect Methyl II Complete PCR Array is tested against predetermined specifications to ensure consistent product quality.

Product Use Limitations

Purchaser agrees that use of this product and data therefrom is limited solely to the purchaser and for only the purchaser's own internal molecular biology research applications ("Permitted Use"), and shall not be re-sold or used for any other purposes (all of which are expressly prohibited), including without limitation diagnostic purposes, uses that could require regulatory approval for diagnostics from an agency of any government or regulatory entity anywhere in the world, diagnosis, prevention, or treatment of disease, and the right to perform commercial services of any kind, including without limitation, reporting the results of purchaser's activities, including without limitation, for a fee or other commercial consideration. Except for the Permitted Use, no rights, titles, or interests in or to any tangible or intangible property rights are conveyed or shall be deemed conveyed by implication, estoppel or otherwise. The performance characteristics of the product other than for the Permitted Use are unknown.

EpiTect Methyl II PCR Arrays are intended for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Technical Assistance

At QIAGEN, we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in sample and assay technologies and the use of QIAGEN products. If you have any questions or experience any difficulties regarding the EpiTect Methyl II Signature Array, EpiTect Methyl II Complete PCR Array, or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information, please see our Technical Support Center at www.qiagen.com/Support or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/Support/MSDS.aspx where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

Introduction

The EpiTect Methyl II PCR System is an innovative technology enabling fast and accurate CpG island DNA methylation profiling of individual genes, as well as disease- and pathway-focused gene panels. The technology provides ready-to-use, predesigned primer assays with high specificity and amplification effeciency to detect the methylation status of the promoter region (gene) of interest. Arrays are disease- or pathway- focused and enable detection of the methylation status of 22 or 94 genes simultaneously. EpiTect Methyl II PCR Arrays use the MethylScreen Technology provided under license from Orion Genomics, LLC.

Approximately 60–70% of all human gene promoters overlap with CpG islands — regions with an elevated GC content and a high frequency of CpG dinucleotides. Gene silencing by means of DNA methylation of specific gene promoters is a well-known feature of neoplastic cells and plays an important role in normal cell differentiation and development (1). DNA methylation occurs mainly at CpG dinucleotides and involves the enzymatic addition of a methyl group to the cytosine residue, without changing the primary DNA sequences.

Such modifications at regulatory regions (in particular gene promoters), correlate well with the transcriptional state of a gene: DNA methylation represses transcription while DNA unmethylation can lead to increased transcription levels. DNA methylation is an essential mechanism for normal cellular development, imprinting, X-chromosome inactivation, and maintaining tissue specificity. It can also contribute significantly to the progression of various human diseases.

The profiling of tumor suppressor genes and other key genes allows the correlation of CpG island methylation status with transcriptional status, biological phenotypes, or disease outcomes. Therefore, the results can provide insights into the molecular mechanisms and biological pathways and aid in the discovery and development of biomarkers.

Principle and procedure

The method employed by the EpiTect Methyl II PCR System is based on detection of remaining input DNA after cleavage with a methylation-sensitive and/or a methylation-dependent restriction enzyme (2). These enzymes will digest unmethylated and methylated DNA, respectively. Following digestion, the remaining DNA in each individual enzyme reaction is quantified by real-time PCR using primers that flank a promoter (gene) region of interest. The relative fractions of methylated and unmethylated DNA are subsequently determined by comparing the amount in each digest with that of a mock (no enzymes added) digest using a ΔC_T method. The reliability and simplicity of the procedure make this technology highly suited for semi-high–throughput DNA methylation profiling and biomarker development for various research fields, such as stem cell differentiation and development.

A protocol overview of the EpiTect Methyl II PCR Array is shown in Figure 1. Briefly, input genomic DNA is aliquoted into four equal portions and subjected to mock (no enzyme), methylation-sensitive (MSRE), methylation-dependent (MDRE), and double (MSRE and MDRE) restriction endonuclease digestion. After digestion, the enzyme reactions are mixed directly with qPCR master mix and are dispensed into a PCR Array plate containing pre-aliquoted primer mixes. Real-time PCR is carried out using specified cycling conditions. Finally, the raw ΔC_T values are pasted into the data analysis spreadsheet, which automatically calculates the relative amount of methylated and unmethylated DNA fractions.

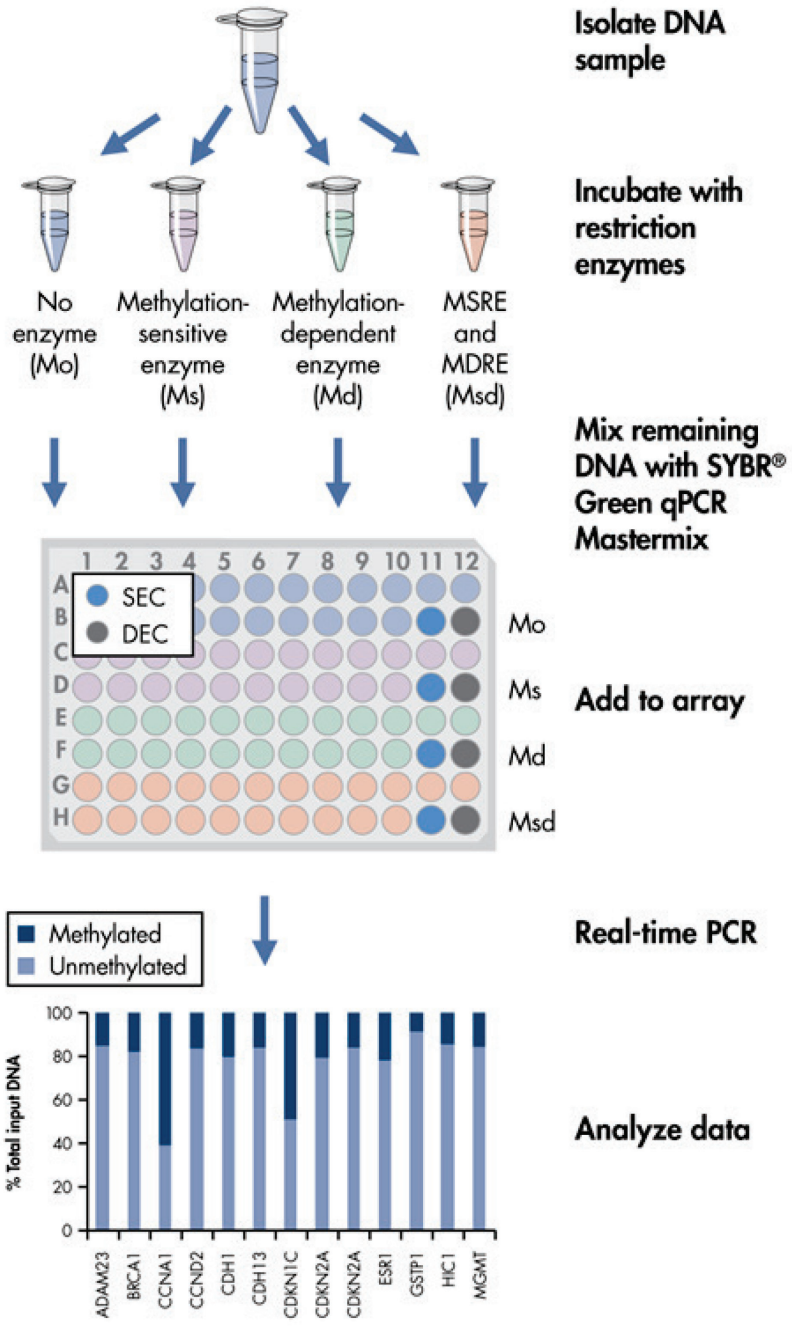


Figure 1. EpiTect Methyl II PCR procedure.

The product of the mock (no enzyme) digestion represents the total amount of input DNA for real-time PCR detection. In the methylation-sensitive digestion (Ms) reaction, the MSRE will digest unmethylated and partially methylated DNA. The remaining hypermethylated DNA — DNA in which all CpG sites are methylated — will be detected by real-time PCR. In the methylation-dependent digestion (Md) reaction, the MDRE will preferentially digest methylated DNA. The remaining unmethylated DNA will be detected by real-time PCR. In the double digestion (Msd) reaction, both enzymes are present, and all DNA molecules (both methylated and unmethylated) will be digested. This reaction measures the background and the fraction of input DNA refractory to enzyme digestion.

EpiTect Methyl II PCR Arrays provide gene methylation status as percentage unmethylated (UM) and percentage methylated (M) fraction of input DNA. Unmethylated represents the fraction of input genomic DNA containing no methylated CpG sites in the amplified region of a gene. Methylated represents fraction of input genomic DNA containing two or more methylated CpG sites in the targeted region of a gene.

Each well of EpiTect Methyl II PCR Array plate contains a different primer assay mixed with an inert dye. This dye does not affect assay performance or fluorescence detection.

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate material safety data sheets (MSDSs), available from the product supplier.

- DNeasy® Blood and Tissue Kit (cat. nos. 69504 or 69506) or the AllPrep® DNA/RNA Mini Kit (cat. no. 80204) for preparation of DNA
- EpiTect Methyl II DNA Restriction Kit (cat. no. 335452)

  IMPORTANT: The EpiTect Methyl II DNA Restriction Kit contains all necessary components for cleavage of methylated and unmethylated DNA and is essential for a complete and successful experiment. The reagents included in the kit are sufficient for processing up to 12 µg of genomic DNA. The actual number of samples processed is dependent on the number of genes analyzed and the plate format selected.
- Appropriate RT² SYBR® Green qPCR Mastermix (be sure to select the correct format for the PCR instrument, and size and quantity for the number of samples):

  RT² SYBR Green ROX™ qPCR Mastermix (cat. nos. 330520, 330522, 330523, 330521, 330529) for Applied Biosystems, Stratagene, and Eppendorf Mastercycler ep realplex instruments with a ROX filter set

  RT² SYBR Green Fluor qPCR Mastermix (cat. nos. 330510, 330512, 330513, 330511, 330519) for Bio-Rad iCycler, MyiQ, MyiQ2, and iQ 5 instruments

  RT² SYBR Green qPCR Mastermix (cat. nos. 330500, 330502, 330503, 330501, 330509) for instruments that do not require a reference dye, e.g., Bio-Rad models CFX96, CFX384, Bio-Rad/MJ Research Opticon 2, Bio-Rad/MJ Research Chromo4; Roche LightCycler 480 (96- and 384-well)
- RT² PCR Array Loading Reservoirs (cat. no. 338162)
- Real-time PCR instrument (see list of supported instruments on page 5)
- Calibrated single- and multi-channel pipets
- RNase-/DNase-free pipet tips and tubes
- RNase-/DNase-free 100 µl regular PCR tubes (8- or 12-tube strings)
- Molecular biology grade RNase- and DNase-free water

Important Notes

Controls for monitoring enzyme digestion efficiency

A successful EpiTect Methyl II PCR experiment relies on efficient DNA digestion by methylation-sensitive and methylation-dependent restriction enzymes. Each EpiTect Methyl II PCR Array includes specific control assays for monitoring the cutting efficiencies of these enzymes and ensuring reliable and reproducible results. After the C_T values are pasted into the Microsoft® Excel® data analysis spreadsheet, a "Pass" or "Fail" result is returned for the SEC (methylation sensitive enzyme control) and DEC (methylation dependent enzyme control) assays.

DNA contamination

For reliable results, it is very important to prevent contamination of the EpiTect Methyl II PCR assay reactions with foreign DNA. Even very small amounts of foreign DNA can artificially inflate SYBR Green signals, yielding false positive results. The most common source of contamination in the PCR reagents comes from the products of previous PCR experiments in your working area. To minimize contamination, follow the recommendations below:

- Wear gloves throughout the entire procedure
- Use only fresh PCR-grade reagents and labware
- Physically separate the workspace for PCR setup and post-PCR work
- Never open PCR plates or tubes after real-time PCR instrument runs
- Before setting up an experiment, decontaminate the PCR workspace and labware (pipet barrels, tube racks, etc.) with 10% bleach and UV light. Preferentially set up reactions in a PCR workstation.
- Do not remove the protective film from the PCR array until immediately before use
- Close all tubes containing PCR products as soon as possible after use
- Treat any labware (tips or tubes) containing PCR products or other DNA with 10% bleach before discarding

Genomic DNA preparation

High-quality DNA is a prerequisite for a successful EpiTect Methyl II PCR assay reaction. Therefore, sample handling and genomic DNA isolation procedures are crucial to the success of the experiment. Residual traces of proteins, salts, or other contaminants will either degrade the DNA or decrease the restriction enzyme activities necessary for optimal DNA digestion.

We recommend the DNeasy Blood and Tissue Kit or the AllPrep DNA/RNA Mini Kit for preparation of genomic DNA samples. When using the DNeasy Blood and Tissue Kit, ensure that samples have been treated for the removal of RNA, as RNA contamination will cause inaccuracies in DNA concentration measurements and may affect restriction digestion efficiency. Do not omit the recommended RNase treatment step to remove RNA. If genomic DNA samples are harvested from biological samples where purification kits are not available, contact QIAGEN Technical Services for suggestions.

For best results, resuspend or dilute all DNA samples in DNase-free water, or alternatively, in DNase-free 10 mM Tris buffer pH 8.0 without EDTA.

Measurement of DNA concentration and purity by UV spectrophotometry

Prepare dilutions of genomic DNA samples and measure absorbance in DNase-free 10 mM Tris buffer, pH 8.0. The spectral properties of nucleic acids are highly dependent on pH. The recommended ratios and values for DNA are as follows:

- A_{260}/A_{230}: >1.7
- A_{260}/A_{280}: >1.8
- A_{260} concentration: >4 µg/ml

DNA concentrations for restriction digestion and PCR assay

Using the recommended amount of DNA optimizes the sensitivity of detecting methylated DNA. More input DNA may be used if analyzing hypermethylated DNA isolated from samples of heterogeneous cell types, e.g., tumor samples, where heavy non-tumor cell contamination is expected, e.g., blood, stromal cells, etc. However, maintain the specific enzyme to DNA ratios outlined below for each assay, and purchase additional qPCR plates to ensure assay consistency.

Using the EpiTect Methyl II DNA Restriction Kit

- **IMPORTANT**: Do not vortex enzymes

  Methylation-dependent enzyme B is very sensitive to vortexing. Extensive vortexing may cause a loss of enzyme activity. Mix enzymes by pipetting gently up and down.

- Store enzymes at –20°C. When in use, enzymes should be kept on ice.

Protocol: EpiTect Methyl II Signature PCR Array for 22 Genes in a 96-well Format and 1 DNA Sample

Be sure to read "Important Notes", page 12, before starting the protocol.

Procedure

Restriction digestion

1. **Perform the restriction digestions using the EpiTect Methyl II DNA Restriction Kit (cat. no. 335452).**
2. **Prepare a reaction mix without enzymes as indicated in Table 1.**
   We recommend using 1 µg genomic DNA. 5x Restriction Digestion Buffer should be thawed and vortexed well before use. If any precipitates are present in the buffer, continue mixing the buffer until precipitates dissolve.

Table 1. Reaction mix without enzymes

Component	Volume
Genomic DNA (1 µg)	Variable
5x Restriction Digestion Buffer	26 µl
RNase-/DNase-free water	Variable
Final volume	**120 µl**

3. **Add RNase-/DNase-free water to make the final volume 120 µl. Vortex to thoroughly mix the components and centrifuge briefly in a microcentrifuge..**
4. **Set up 4 digestion reactions (M_o, M_s, M_d, and M_{sd}) according to Table 2.**
   IMPORTANT: All 4 tubes must contain equal amounts of genomic DNA.

Table 2. Restriction digestion

Component	M_o	M_s	M_d	M_{sd}
Reaction mix from step 3	28 µl	28 µl	28 µl	28 µl
Methylation-sensitive enzyme A	–	1 µl	–	1 µl
Methylation-dependent enzyme B	–	–	1 µl	1 µl
RNase-/DNase-free water	2 µl	1 µl	1 µl	–
Final volume	**30 µl**	**30 µl**	**30 µl**	**30 µl**

5. **Pipet up and down to gently, but thoroughly mix the components. Centrifuge the tubes briefly in a microcentrifuge.**
   IMPORTANT: Do not vortex!
6. **Incubate all 4 tubes at 37°C for 6 h in a heating block or thermal cycler. The reaction can also be performed overnight.**
7. **After incubation, stop the reactions by heat-inactivating the enzymes at 65°C for 20 min.**
8. **The reactions are now ready for use or storage at –20°C. Mix the samples thoroughly by vortexing before use. Centrifuge the samples briefly in a microcentrifuge and proceed to step 1 of "Setting up the PCR".**

Setting up the PCR

1. **Prepare a reaction for each of the 4 digestions (M_o, M_s, M_d, and M_{sd}) in a 1.5 ml tube according to Table 3.**

Table 3. PCR setup

Component	M_o	M_s	M_d	M_{sd}
PCR master mix	330 µl	330 µl	330 µl	330 µl
M_o digest	30 µl	–	–	–
M_s digest	–	30 µl	–	–
M_d digest	–	–	30 µl	–
M_{sd} digest	–	–	–	30 µl
RNase-/DNase-free water	300 µl	300 µl	300 µl	300 µl
Final volume	**660 µl**	**660 µl**	**660 µl**	**660 µl**

2. **Mix tubes well by vortexing, and briefly centrifuge the contents to the bottom of the tube.**
3. **Add 25 µl of the M_o reaction to each well in rows A and B of the 96-well EpiTect Methyl II Signature PCR Array. Add 25 µl of the M_s reaction to each well in rows C and D. Add 25 µl of the M_d reaction to each well in rows E and F. Finally, add 25 µl of the M_{sd} reaction to each well in rows G and H, as shown in Figure 2.**

Digest	Well	1	2	3	4	5	6	7	8	9	10	11	12
M_o	A	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
	B	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	SEC	DEC
M_s	C	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
	D	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	SEC	DEC
M_d	E	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
	F	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	SEC	DEC
M_{sd}	G	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
	H	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	SEC	DEC

Figure 2. Layout of a 96-well EpiTect Methyl II Signature PCR Array.

4. **Seal or cap the wells of the plate. Centrifuge the plate for 1 min at 2000 rpm to remove any air bubbles.**

Running the PCR

1. **Program the thermal cycler according to the manufacturer's instructions, using the conditions outlined in Table 4.**

   Note: It is critical that the cycling conditions are followed exactly.

Table 4. PCR cycling protocol

Temperature	Time	Number of cycles
95°C	10 min*	1 cycle
99°C 72°C	30 s 1 min	3 cycles
97°C 72°C	15 s 1 min†	40 cycles
According to instrument recommendations	Melting curve segment	

* Hot-start to activate DNA polymerase.

† Detect and record SYBR Green fluorescence from each well during the annealing step of each cycle.

2. **After the run has finished, analyze the data as described in "Data Analysis", page 30.**

Protocol: EpiTect Methyl II Complete PCR Array for 94 Genes in a 96-well format and 1 DNA Sample

Be sure to read "Important Notes", page 12, before starting the protocol.

Procedure

Restriction digestion

1. **Perform the restriction digestions using the EpiTect Methyl II DNA Restriction Kit (cat. no. 335452).**
2. **Prepare a reaction mix without enzymes as indicated in Table 5.**
   We recommend using 4 µg genomic DNA. 5x Restriction Digestion Buffer should be thawed and vortexed well before use. If any precipitates are present in the buffer, continue mixing the buffer until precipitates dissolve.

Table 5. Reaction mix without enzymes

Component	Volume
Genomic DNA (4 µg)	Variable
5x Restriction Digestion Buffer	100 µl
RNase-/DNase-free water	Variable
Final volume	**470 µl**

3. **Add RNase-/DNase-free water to make the final volume 470 µl. Vortex to thoroughly mix the components and centrifuge briefly in a microcentrifuge.**
4. **Set up 4 digestion reactions (M_o, M_s, M_d, and M_{sd}) according to Table 6.**
   IMPORTANT: All 4 tubes must contain equal amounts of genomic DNA.

Table 6. Restriction digestion

Component	M_o	M_s	M_d	M_{sd}
Reaction mix from step 3	112 µl	112 µl	112 µl	112 µl
Methylation-sensitive enzyme A	–	4 µl	–	4 µl
Methylation-dependent enzyme B	–	–	4 µl	4 µl
RNase-/DNase-free water	8 µl	4 µl	4 µl	–
Final volume	**120 µl**	**120 µl**	**120 µl**	**120 µl**

5. **Pipet up and down to gently, but thoroughly mix the components. Centrifuge the tubes briefly in a microcentrifuge.**
   IMPORTANT: Do not vortex!
6. **Incubate all 4 tubes at 37°C for 6 h in a heating block or thermal cycler. The reaction can also be performed overnight.**
7. **After incubation, stop the reactions by heat-inactivating the enzymes at 65°C for 20 min.**
8. **The reactions are now ready for use or storage at –20°C. Mix the samples thoroughly by vortexing before use. Centrifuge the samples briefly and proceed to step 1 of "Setting up the PCR".**

Setting up the PCR

1. **Prepare a reaction for each of the 4 digestions (M_o, M_s, M_d, and M_{sd}) in a 15 ml tube according to Table 7.**

Table 7. PCR setup

Component	M_o	M_s	M_d	M_{sd}
PCR master mix	1280 µl	1280 µl	1280 µl	1280 µl
M_o digest	120 µl	–	–	–
M_s digest	–	120 µl	–	–
M_d digest	–	–	120 µl	–
M_{sd} digest	–	–	–	120 µl
RNase-/DNase-free water	1160 µl	1160 µl	1160 µl	1160 µl
Final volume	**2560 µl**	**2560 µl**	**2560 µl**	**2560 µl**

2. **Mix tubes well by vortexing, and briefly centrifuge the contents to the bottom of the tube.**
3. **Add 25 µl of the M_o reaction to each well in rows A and B of the four 96-well EpiTect Methyl II Complete PCR Arrays. Add 25 µl of the M_s reaction to each well in rows C and D. Add 25 µl of the M_d reaction to each well in rows E and F. Finally, add 25 µl of the M_{sd} reaction to each well in rows G and H, as shown in Figure 3.**

Digest	Well	1	2	3	4	5	6	7	8	9	10	11	12
M_o	A	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
	B	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	SEC	DEC
M_s	C	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
	D	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	SEC	DEC
M_d	E	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
	F	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	SEC	DEC
M_{sd}	G	G01	G02	G03	G04	G05	G06	G07	G08	G09	G10	G11	G12
	H	G13	G14	G15	G16	G17	G18	G19	G20	G21	G22	SEC	DEC

Figure 3. Layout of 96-well EpiTect Methyl II Complete PCR Array. The first of four 96-well plates is laid out as shown. On the remaining 3 plates, the SEC and DEC assays are replaced by gene-specific primers (G45, G69, G93 and G46, G70, G94, respectively).

4. **Seal or cap the wells of each plate. Centrifuge the plates for 1 min at 2000 rpm to remove any air bubbles.**

   Note: One plate can be run immediately and the other 3 plates placed at –20°C until the PCR instrument is ready for another run. Do not thaw the plates before running the PCR, but place them directly in the PCR instrument.

Running the PCR

1. **Program the thermal cycler according to the manufacturer's instructions, using the conditions outlined in Table 8.**

   Note: It is critical that the cycling conditions are followed exactly.

Table 8. PCR cycling protocol

Temperature	Time	Number of cycles
95°C	10 min*	1 cycle
99°C 72°C	30 s 1 min	3 cycles
97°C 72°C	15 s 1 min†	40 cycles
According to instrument recommendations	Melting curve segment	

* Hot-start to activate DNA polymerase.

† Detect and record SYBR Green fluorescence from each well during the annealing step of each cycle.

2. **After the run has finished, analyze the data as described in "Data Analysis", page 30.**

Protocol: EpiTect Methyl II Complete PCR Array for 94 Genes in a 384-well Format and 1 DNA Sample

Be sure to read "Important Notes", page 12, before starting the protocol.

Procedure

Restriction digestion

1. **Perform the restriction digestions using the EpiTect Methyl II DNA Restriction Kit (cat. no. 335452).**
2. **Prepare a reaction mix without enzymes as indicated in Table 9.**
   We recommend using 2 µg genomic DNA. The 5x Restriction Digestion Buffer should be thawed and vortexed well before use. If any precipitates are present in the buffer, continue mixing the buffer until precipitates dissolve.

Table 9. Reaction mix without enzymes

Component	Volume
Genomic DNA (2 µg)	Variable
5x Restriction Digestion Buffer	100 µl
RNase-/DNase-free water	Variable
Final volume	**470 µl**

3. **Add RNase-/DNase-free water to make the final volume 470 µl. Vortex to thoroughly mix the components and centrifuge briefly in a microcentrifuge.**
4. **Set up 4 digestion reactions (M_o, M_s, M_d, and M_{sd}) according to Table 10.**
   IMPORTANT: All 4 tubes must contain equal amounts of genomic DNA.

Table 10. Restriction digestion

Component	M_o	M_s	M_d	M_{sd}
Reaction mix from step 3	116 µl	116 µl	116 µl	116 µl
Methylation-sensitive enzyme A	–	2 µl	–	2 µl
Methylation-dependent enzyme B	–	–	2 µl	2 µl
RNase-/DNase-free water	4 µl	2 µl	2 µl	–
Final volume	**120 µl**	**120 µl**	**120 µl**	**120 µl**

5. **Pipet up and down to gently, but thoroughly mix the components. Centrifuge the tubes briefly in a microcentrifuge.**
   IMPORTANT: Do not vortex!
6. **Incubate all 4 tubes at 37°C for 6 h in a heating block or thermal cycler. The reaction can also be performed overnight.**
7. **After incubation, stop the reactions by heat-inactivating the enzymes at 65°C for 20 min.**
8. **The reactions are now ready for use or storage at –20°C. Mix the samples thoroughly by vortexing before use. Centrifuge the samples down briefly and proceed to step 1 of "Setting up the PCR".**

Setting up the PCR

1. **Prepare a reaction for each of the 4 digestions (M_o, M_s, M_d, and M_{sd}) in a 1.5 ml tube according to Table 11.**

Table 11. PCR setup

Component	M_o	M_s	M_d	M_{sd}
PCR master mix	590 µl	590 µl	590 µl	590 µl
M_o digest	120 µl	–	–	–
M_s digest	–	120 µl	–	–
M_d digest	–	–	120 µl	–
M_{sd} digest	–	–	–	120 µl
RNase-/DNase-free water	470 µl	470 µl	470 µl	470 µl
Final volume	**1180 µl**	**1180 µl**	**1180 µl**	**1180 µl**

2. **Mix tubes well by vortexing, and briefly centrifuge the contents to the bottom of the tube.**
3. **Carefully add each reaction mix to the appropriate wells of the EpiTect Methyl II Complete PCR Array 384-well plate as follows, using the provided 384EZLoad Covers (Figure 4, next page).**

   Place Cover 1 on the plate. Add 10 µl M_o reaction to the open wells (odd number wells of rows A, C, E, G, I, K, M, and O). Remove and discard the cover.

   Place Cover 2 on the plate. Add 10 µl M_s reaction to the open wells (even number wells of rows A, C, E, G, I, K, M, and O). Remove and discard the cover.

   Place Cover 3 on the plate. Add 10 µl M_d reaction to the open wells (odd number wells of rows B, D, F, H, J, L, N, and P). Remove and discard the cover.

   Place Cover 4 on the plate. Add 10 µl M_{sd} reaction to the open wells (even number wells of rows B, D, F, H, J, L, N, and P). Remove and discard the cover.

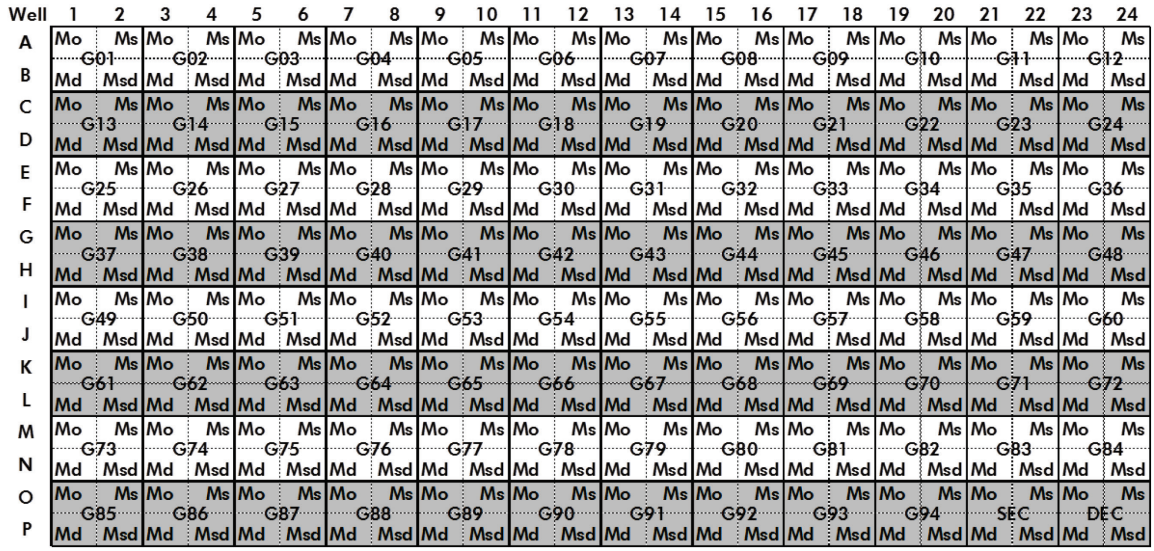

Well 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
A Mo Ms G01 Md Msd Mo Ms G02 Md Msd Mo Ms G03 Md Msd Mo Ms G04 Md Msd Mo Ms G05 Md Msd Mo Ms G06 Md Msd Mo Ms G07 Md Msd Mo Ms G08 Md Msd Mo Ms G09 Md Msd Mo Ms G10 Md Msd Mo Ms G11 Md Msd Mo Ms G12 Md Msd
B
C Mo Ms G13 Md Msd Mo Ms G14 Md Msd Mo Ms G15 Md Msd Mo Ms G16 Md Msd Mo Ms G17 Md Msd Mo Ms G18 Md Msd Mo Ms G19 Md Msd Mo Ms G20 Md Msd Mo Ms G21 Md Msd Mo Ms G22 Md Msd Mo Ms G23 Md Msd Mo Ms G24 Md Msd
D
E Mo Ms G25 Md Msd Mo Ms G26 Md Msd Mo Ms G27 Md Msd Mo Ms G28 Md Msd Mo Ms G29 Md Msd Mo Ms G30 Md Msd Mo Ms G31 Md Msd Mo Ms G32 Md Msd Mo Ms G33 Md Msd Mo Ms G34 Md Msd Mo Ms G35 Md Msd Mo Ms G36 Md Msd
F
G Mo Ms G37 Md Msd Mo Ms G38 Md Msd Mo Ms G39 Md Msd Mo Ms G40 Md Msd Mo Ms G41 Md Msd Mo Ms G42 Md Msd Mo Ms G43 Md Msd Mo Ms G44 Md Msd Mo Ms G45 Md Msd Mo Ms G46 Md Msd Mo Ms G47 Md Msd Mo Ms G48 Md Msd
H
I Mo Ms G49 Md Msd Mo Ms G50 Md Msd Mo Ms G51 Md Msd Mo Ms G52 Md Msd Mo Ms G53 Md Msd Mo Ms G54 Md Msd Mo Ms G55 Md Msd Mo Ms G56 Md Msd Mo Ms G57 Md Msd Mo Ms G58 Md Msd Mo Ms G59 Md Msd Mo Ms G60 Md Msd
J
K Mo Ms G61 Md Msd Mo Ms G62 Md Msd Mo Ms G63 Md Msd Mo Ms G64 Md Msd Mo Ms G65 Md Msd Mo Ms G66 Md Msd Mo Ms G67 Md Msd Mo Ms G68 Md Msd Mo Ms G69 Md Msd Mo Ms G70 Md Msd Mo Ms G71 Md Msd Mo Ms G72 Md Msd
L
M Mo Ms G73 Md Msd Mo Ms G74 Md Msd Mo Ms G75 Md Msd Mo Ms G76 Md Msd Mo Ms G77 Md Msd Mo Ms G78 Md Msd Mo Ms G79 Md Msd Mo Ms G80 Md Msd Mo Ms G81 Md Msd Mo Ms G82 Md Msd Mo Ms G83 Md Msd Mo Ms G84 Md Msd
N
O Mo Ms G85 Md Msd Mo Ms G86 Md Msd Mo Ms G87 Md Msd Mo Ms G88 Md Msd Mo Ms G89 Md Msd Mo Ms G90 Md Msd Mo Ms G91 Md Msd Mo Ms G92 Md Msd Mo Ms G93 Md Msd Mo Ms G94 Md Msd Mo Ms SEC Md Msd Mo Ms DEC Md Msd
P

Figure 4. Layout of 384-well EpiTect Methyl II Complete PCR Array.

4. **Seal or cap the wells of the plate. Centrifuge the plate for 1 min at 2000 rpm to remove any air bubbles.**

Running the PCR

1. **Program the thermal cycler according to the manufacturer's instructions, using the conditions outlined in Table 12.**

   Note: It is critical that the cycling conditions are followed exactly.

Table 12. PCR cycling protocol

Temperature	Time	Number of cycles
95°C	10 min*	1 cycle
99°C 72°C	30 s 1 min	3 cycles
97°C 72°C	15 s 1 min†	40 cycles
According to instrument recommendations	Melting curve segment	

* Hot-start to activate DNA polymerase.

† Detect and record SYBR Green fluorescence from each well during the annealing step of each cycle.

2. **After the run has finished, analyze the data as described in "Data Analysis", page 30.**

Protocol: EpiTect Methyl II Signature PCR Array for 22 Genes in a 384-well Format and 4 DNA Samples

Be sure to read "Important Notes", page 12, before starting the protocol.

Procedure

Restriction digestion

1. **Perform the restriction digestions using the EpiTect Methyl II DNA Restriction Kit (cat. no. 335452).**
2. **Prepare a reaction mix without enzymes as indicated in Table 13.**

   We recommend using 0.5 µg genomic DNA per sample. The amounts shown in Table 13 are for one sample. Repeat this process for each sample. 5x Restriction Digestion Buffer should be thawed and vortexed well before use. If any precipitates are present in the buffer, continue mixing the buffer until precipitates dissolve.

Table 13. Reaction mix without enzymes

Component	Volume
Genomic DNA (0.5 µg)	Variable
5x Restriction Digestion Buffer	26 µl
RNase-/DNase-free water	Variable
Final volume	**125 µl**

3. **Add RNase-/DNase-free water to make the final volume 125 µl. Vortex to thoroughly mix the components and centrifuge briefly in a microcentrifuge.**
4. **Set up 4 digestion reactions (M_o, M_s, M_d, and M_{sd}) for each sample according to Table 14.**

   IMPORTANT: All 4 tubes must contain equal amounts of genomic DNA.

Table 14. Restriction digestion

Component	M_o	M_s	M_d	M_{sd}
Reaction mix from step 3	28 µl	28 µl	28 µl	28 µl
Methylation-sensitive enzyme A	–	1 µl	–	1 µl
Methylation-dependent enzyme B	–	–	1 µl	1 µl
RNase-/DNase-free water	2 µl	1 µl	1 µl	–
Final volume	**30 µl**	**30 µl**	**30 µl**	**30 µl**

5. **Pipet up and down to gently, but thoroughly mix the components. Centrifuge the tubes briefly in a microcentrifuge.**
   IMPORTANT: Do not vortex!
6. **Incubate all 4 tubes at 37°C for 6 h in a heating block or thermal cycler. The reaction can also be performed overnight.**
7. **After incubation, stop the reactions by heat-inactivating the enzymes at 65°C for 20 min.**
8. **The reactions are now ready for use or storage at –20°C. Mix the samples thoroughly by vortexing before use. Spin the samples down briefly and proceed to step 1 of "Setting up the PCR".**

Setting up the PCR

1. **Prepare a reaction for each of the 4 digestions (M_o, M_s, M_d, and M_{sd}) in a 1.5 ml tube according to Table 15.**
   The volumes shown in Table 15 are for one sample.

Table 15. PCR setup

Component	M_o	M_s	M_d	M_{sd}
PCR master mix	170 µl	170 µl	170 µl	170 µl
M_o digest	30 µl	–	–	–
M_s digest	–	30 µl	–	–
M_d digest	–	–	30 µl	–
M_{sd} digest	–	–	–	30 µl
RNase-/DNase-free water	140 µl	140 µl	140 µl	140 µl
Final volume	**340 µl**	**340 µl**	**340 µl**	**340 µl**

2. **Mix tubes well by vortexing, and briefly centrifuge the contents to the bottom of the tube.**
3. **Carefully add each reaction mix to the appropriate wells of the EpiTect Methyl II Signature PCR Array 384-well plate as follows, using the provided 384EZLoad Covers (Figure 4, next page).**

   Place Cover 1 on the plate. Add 10 µl M_o reaction to the open odd numbered wells of rows A and C for sample 1, rows E and G for sample 2, rows I and K for sample 3, and rows M and O for sample 4. Remove and discard the cover.

   Place Cover 2 on the plate. Add 10 µl M_s reaction to the open even numbered wells of rows A and C for sample 1, rows E and G for sample 2, rows I and K for sample 3, and rows M and O for sample 4. Remove and discard the cover.

   Place Cover 3 on the plate. Add 10 µl M_d reaction to the open odd numbered wells of rows B and D for sample 1, rows F and H for sample 2, rows J and L for sample 3, and rows N and P for sample 4. Remove and discard the cover.

   Place Cover 4 on the plate. Add 10 µl M_{sd} reaction to the open even numbered wells of rows B and D for sample 1, rows F and H for sample 2, rows J and L for sample 3, and rows N and P for sample 4. Remove and discard the cover.

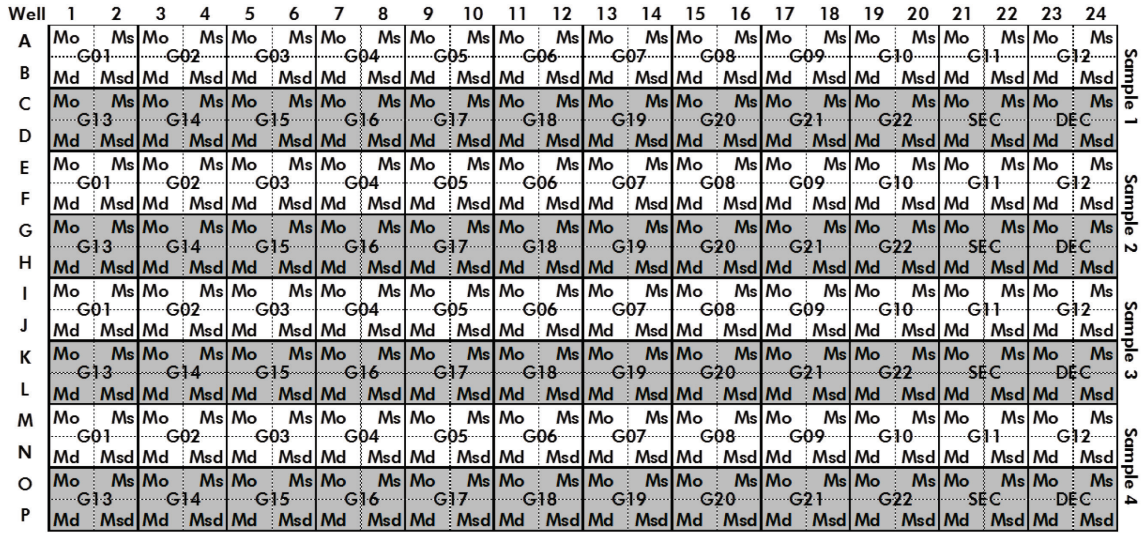

Well 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24
A Mo Ms G01 Md Msd G02 G03 G04 G05 G06 G07 G08 G09 G10 G11 G12
B
C Mo Ms G13 Md Msd G14 G15 G16 G17 G18 G19 G20 G21 G22 SEC DEC
D
E Mo Ms G01 Md Msd G02 G03 G04 G05 G06 G07 G08 G09 G10 G11 G12
F
G Mo Ms G13 Md Msd G14 G15 G16 G17 G18 G19 G20 G21 G22 SEC DEC
H
I Mo Ms G01 Md Msd G02 G03 G04 G05 G06 G07 G08 G09 G10 G11 G12
J
K Mo Ms G13 Md Msd G14 G15 G16 G17 G18 G19 G20 G21 G22 SEC DEC
L
M Mo Ms G01 Md Msd G02 G03 G04 G05 G06 G07 G08 G09 G10 G11 G12
N
O Mo Ms G13 Md Msd G14 G15 G16 G17 G18 G19 G20 G21 G22 SEC DEC
P
Sample 1
Sample 2
Sample 3
Sample 4

Figure 5. Layout of 384-well EpiTect Methyl II Signature PCR Array.

4. **Seal or cap the wells of the plate. Centrifuge the plate for 1 min at 2000 rpm to remove any air bubbles.**

Running the PCR

1. **Program the thermal cycler according to the manufacturer's instructions, using the conditions outlined in Table 16.**

   Note: It is critical that the cycling conditions are followed exactly.

Table 16. PCR cycling protocol

Temperature	Time	Number of cycles
95°C	10 min*	1 cycle
99°C 72°C	30 s 1 min	3 cycles
97°C 72°C	15 s 1 min†	40 cycles
According to instrument recommendations	Melting curve segment	

* Hot-start to activate DNA polymerase.

† Detect and record SYBR Green fluorescence from each well during the annealing step of each cycle.

2. **After the run has finished, analyze the data as described in "Data Analysis", page 30.**

Data Analysis

Obtaining raw threshold cycle (C_T) values

After the cycling program has completed, obtain the C_T values according to the instructions provided by the manufacturer of the real-time PCR instrument. We recommend manually setting the baseline and threshold values as follows.

Note: when comparing multiple plates, make sure that the settings for all plates are identical.

Baseline: Using the Linear View of the amplification plots, set the instrument to use the readings from cycle number 2 through the cycle just before the earliest visible amplification, usually between cycle 10 and 15.

Threshold value: Using the Log View of the amplification plots, place the threshold above the background signal but within the lower third of the linear portion of the amplification curves.

Exporting C_T values

Export and/or copy/paste the C_T values from the instrument software to a blank Microsoft Excel spreadsheet according the manufacturer's instructions for the real-time PCR instrument.

Microsoft Excel based data analysis template

First, download the EpiTect Methyl II PCR Array Microsoft Excel based data analysis template, which is available at: www.sabiosciences.com/dna_methylation_data_analysis.php.

Then, paste in the C_T value data and analyze the automatically generated results by following the directions in the "Instructions" worksheet of the Excel file.

Data quality control

Mock digest (M_o) C_T values

The C_T values of the mock digests for all genes should be within the range of 18 to 27 cycles, if the recommended amounts of genomic DNA were used.

Single enzyme digest (M_s and M_d) C_T values

The C_T values of the M_s and M_d digests should be between the values of the mock and double digests, depending on the methylation status of the DNA samples.

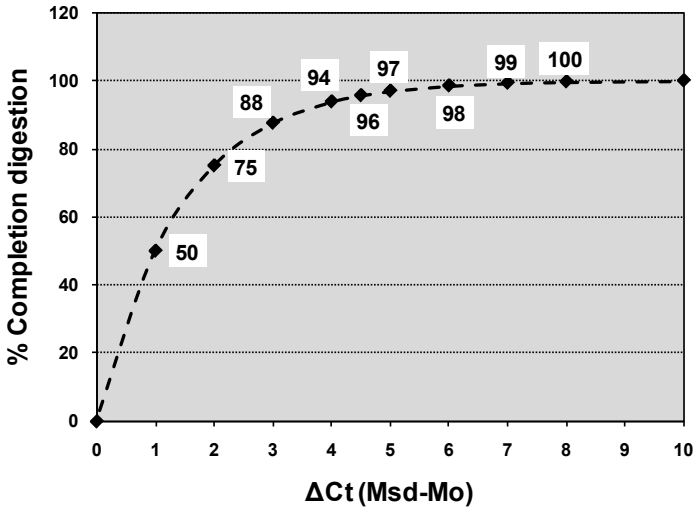
Double digest (M_{sd}) C_T values

The C_T values of the double digests should be higher than the C_T values of the mock digest.

Enzyme digestion efficiency

The difference in C_T values between the double digest and mock digest samples represents the analytical window (W) of the assay and should be greater than 3 (ΔC_T [M_{sd} – M_o] > 3). This means that more than 87.5% of all DNA molecules in the samples were digested, and that the assay results are reliable and meaningful. See also the "Data QC Report" worksheet in the Microsoft Excel data analysis template. For every gene, the analytical window (W) values should be >3 and the F_R values should be <12.5%.

When using the EpiTect Methyl II PCR Assay for EP_SEC (cat. no. EPHS115450-1A) and EP_DEC (cat. no. EPHS115451-1A) to test the cutting efficiencies of the restriction enzymes, copy and paste the C_T values for these controls into the Microsoft Excel data analysis template. For the SEC assay, the difference in C_T values between the methylation-sensitive and mock digests should be equal to or greater than 4 (ΔC_T (M_s – M_o) ≥ 4) to "Pass" the quality control. Likewise for the DEC control assay, the difference in C_T values between the methylation-dependent and mock digests should be equal to or greater than 4 (ΔC_T (M_d – M_o) ≥ 4) to "Pass" the quality control. "Pass" means that more than 93.6% of control DNA molecules spiked in buffer (5x Restriction Digestion Buffer) were digested, showing that the restriction enzymes were active and digested the DNA efficiently. See the "Result" worksheet in the Microsoft Excel data analysis template. Both the SEC and DEC assays should show "Pass" in the analysis.



Dissociation (melting) curve

Perform the default melting curve program on the instrument immediately after the cycling program. Generate the first derivative dissociation curve for each well in each plate using the instrument's software. A single well-defined peak should appear in each well. If your instrument does not have a default melting curve program, run the following program instead:

97°C, 1 min; 55°C, 30 sec (OPTICS OFF)

55–97°C at 2°C/s (OPTICS ON)

Data Interpretation

EpiTect Methyl II PCR Arrays provide gene methylation status as percentage unmethylated (UM) and percentage methylated (M) fraction of input DNA. "UM" represents the fraction of input genomic DNA containing no methylated CpG sites in the amplified region of a gene. "M" represents the fraction of input genomic DNA containing two or more methylated CpG sites in the targeted region of a gene. The number of CpG sites methylated in a targeted region can vary within the fraction of methylated input DNA.

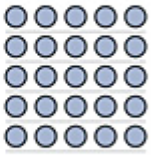
		Final results	
Example #	Input genomic DNA	Unmethylated (UM)	Methylated (M)
1		100%	0%
2		60%	40%
3		0%	100%
4		0%	100%

Figure 6. Pictorial explanations of results.

In the figure above, each horizontal bar represents the targeted region of a gene from one genome. Biological samples usually contain many genomes derived from many cell types. For simplicity, five such genomes are depicted here. Light and dark circles represent unmethylated and methylated CpG sites, respectively.

The "Results" worksheet displays the relative percentage of methylated (M) and unmethylated (UM) DNA in each target genomic DNA sequence. The M values can be used to generate a graphical representation of the data using our developed Hierarchical Clustering method (www.sabiosciences.com/dna_methylation_heatmap.php). Significance of the level of methylated DNA (% of total input DNA) must be defined by the researcher. Methylated DNA may have biological significance if such methylation status is associated with a specific tumor, tissue, or other phenotype. Ideally, to determine if this methylation status is sufficient to repress transcription, measuring the corresponding expression levels and comparing those with the expression levels in the appropriate controls should be considered. Alternatively, results can be compared between a control and experimental DNA samples. Such parallel analysis will allow researchers to see if the methylation status of an experimental sample is substantially different from a matched control sample (i.e., tumor sample vs. normal control or treated sample vs. untreated).

ΔC_T data analysis

Due to the inversely proportional relationship between threshold cycle and the amount of input DNA, and due to the doubling of PCR product with every cycle in the exponential phase of the reaction, the initial DNA amount in each digest before PCR is expressed as:

$$C_{Mo} = 2^{-C_T(M_o)};\ C_{Ms} = 2^{-C_T(M_s)};\ C_{Md} = 2^{-C_T(M_d)};\ C_{Msd} = 2^{-C_T(M_{sd})} \quad (1)$$

The fraction of DNA in each digest is calculated by normalizing the DNA amount to the amount of digestible DNA. The amount of digestible DNA is equal to the total amount of DNA (determined from the mock digest) minus the amount of DNA resistant to DNA digestion (determined from the double digest).

Unmethylated (UM) DNA fraction:

$$F_{UM} = \frac{C_{Md}}{C_{Mo} - C_{Msd}} = \frac{2^{-C_T(M_d)}}{2^{-C_T(M_o)} - 2^{-C_T(M_{sd})}} \quad (2)$$

Hypermethylated (HM) DNA fraction:

$$F_{HM} = \frac{C_{Ms}}{C_{Mo} - C_{Msd}} = \frac{2^{-C_T(M_s)}}{2^{-C_T(M_o)} - 2^{-C_T(M_{sd})}}$$

Intermediately methylated (IM) DNA fraction:

$$F_{IM} = 1 - F_{HM} - F_{UM}$$

Methylated (M) DNA fraction:

$$F_M = F_{HM} + F_{IM} \quad (3)$$

DNA copies resistant (R) to enzyme digestion:

$$F_R = \frac{C_{Msd}}{C_{Mo}} = \frac{2^{-C_T(M_{sd})}}{2^{-C_T(M_o)}} = 2^{-[C_T(M_{sd}) - -C_T(M_o)]} = 2^{-\Delta C_T (M_{sd} - M_o)} \quad (4)$$

Example:

Symbol	**M_o**	**M_s**	**M_d**	**M_{sd}**	**R**	**HM**	**UM**	**M**
CCNA1	23.16	27.11	24.89	36.51	0.0095%	6.47%	30.15%	69.85%

$F_{UM} = 2 \text{ ^ } – (C_T M_d) / (2 \text{ ^ } (– C_T M_o) – 2 \text{ ^ } (– C_T M_{sd})) = 0.3015$ or 30.15%

$F_{HM} = 2 \text{ ^ } – (C_T M_s) / (2 \text{ ^ } (– C_T M_o) – 2 \text{ ^ } (– C_T M_{sd})) = 0.0647$ or 6.47%

$F_{IM} = 1 – F_{HM} – F_{UM} = 1 – 0.0647 – 0.3015 = 0.6338$ or 63.38%

$F_M = F_{HM} + F_{IM} = 0.0647 + 0.6338 = 0.6985$ or 69.85%

$F_R = 2 \text{ ^ } – (C_T M_{sd} – C_T M_o) = 2 \text{ ^ } – (36.51 – 23.16) = 0.0095\%$

Methylation-sensitive or methylation-dependent digest C_T values within one cycle of the mock digest cannot be reliably used to calculate the percentage of either respective methylated DNA fraction. Differences in threshold cycles less than one (1) are within the standard error associated with real-time PCR instruments and experimental procedures. In these situations, the digest with the greatest difference in C_T value from the mock digest is used to calculate its DNA fraction, whether unmethylated or hypermethylated. The opposite fraction (hypermethylated or unmethylated, respectively) is instead calculated as one minus the determined fraction. The amount of intermediately methylated DNA is then assumed to be negligible. Thus, the amount of methylated DNA is represented by the hypermethylated DNA fraction.

If $\Delta C_T (M_s - M_o) < 1.0$ and $\Delta C_T (M_d - M_o) > 1.0$, use following formula to calculate the fraction of hypermethylated DNA:

$$F_M = 1 - F_{UM} = 1 - 2^{-C_T(M_d)} / (2^{-C_T(M_o)} - 2^{-C_T(M_{sd})}) \quad (5)$$

Example:

Symbol	$\mathbf{M_o}$	$\mathbf{M_s}$	$\mathbf{M_d}$	$\mathbf{M_{sd}}$	**R**	**M**	**UM**
CDH13	19.84	20.60	28.38	30.36	0.068%	99.73%	0.27%

$F_{UM} = 2 \text{ ^ } - (C_T M_d) / (2 \text{ ^ } (- C_T M_o) - 2 \text{ ^ } (- C_T M_{sd})) = 0.0027$ or 0.27%

$F_M = F_{HM} = 1 - F_{UM} = 1 - 0.0027 = 0.9973$ or 99.73%

$F_R = 2 \text{ ^ } - (C_T M_{sd} - C_T M_o) = 2 \text{ ^ } - (36.36 - 19.84) = 0.068\%$

If $\Delta C_T (M_d - M_o) < 1.0$ and $\Delta C_T (M_s - M_o) > 1.0$, use following formula to calculate the fraction of unmethylated DNA:

$$F_{UM} = 1 - F_{HM} = 1 - 2^{-C_T(M_s)} / (2^{-C_T(M_o)} - 2^{-C_T(M_{sd})}) \quad (6)$$

Example:

Symbol	$\mathbf{M_o}$	$\mathbf{M_s}$	$\mathbf{M_d}$	$\mathbf{M_{sd}}$	**R**	**M**	**UM**
BRCA1	22.96	32.73	23.34	40.00	0.0007%	0.114%	99.886%

$F_M = F_{HM} = 2 \text{ ^ } - (C_T M_s) / (2 \text{ ^ } (- C_T M_o) - 2 \text{ ^ } (- C_T M_{sd})) = 0.00114$ or 0.114%

$F_{UM} = 1 - F_{HM} = 1 - 0.00114 = 0.99888$ or 99.886%

$F_R = 2 \text{ ^ } - (C_T M_{sd} - C_T M_o) = 2 \text{ ^ } - (40.00 - 22.96) = 0.0007\%$

If both ΔC_T (M_s – M_o) and ΔC_T (M_d – M_o) are less than 1.0, then the fraction of both hypermethylated and unmethylated DNA is assigned as 50%, while again the amount of intermediately methylated DNA is negligible. Thus, the amount of methylated DNA is represented by the hypermethylated DNA fraction.

Example:

Symbol	**M_o**	**M_s**	**M_d**	**M_{sd}**	**R**	**M**	**UM**
SFN	24.03	24.03	24.59	40.00	0.0016%	50.0%	50.0%

$F_M = F_{HM} = F_{UM} = 50.0\%$

$F_R = 2 \text{ ^ } – (C_T M_{sd} – C_T M_o) = 2 \text{ ^ } – (40.00 – 24.03) = 0.0016\%$

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

	Comments and suggestions
Incomplete restriction enzyme digestion: C_T (M_{sd}) – C_T (M_o) < 3 or SEC or DEC "Fail" in the data analysis worksheet	
a) Poor-quality DNA	Repeat the experiment with new DNA samples.
b) Low restriction enzyme activity	Check that the EpiTect Methyl II DNA Restriction Kit has not expired. Be sure to use the correct amount of both enzymes recommended in the protocol for the amount of DNA used. Be sure to mix the enzyme by gently pipetting up and down.
c) RNA contamination in the DNA samples	RNA contamination inhibits restriction enzyme DNA digestion and causes an overestimation of DNA concentration. Be sure to include any RNase treatment steps recommended in the procedure of the chosen DNA preparation kit.
d) Other contaminants in the DNA samples	DNA prepared from difficult organ tissues may contain protein and/or polysaccharide contaminants that significantly inhibit restriction enzyme activity. Organic reagents (such as chloroform, phenol, and isopropanol) used in some DNA kits and protocols may not be completely removed. Be sure to use the recommended DNA isolation kits and protocols and avoid using organic solvent-based methods and protocols for DNA preparation.
e) Too much DNA used in the digestion	Carefully measure the DNA concentration and only use the amount of DNA recommended by the selected PCR setup protocol.

	Comments and suggestions
f) Incorrect incubation conditions	Incubate for at least 4 hours at 37°C and use the size of tubes recommended in the protocol. Use an overnight incubation if a shorter time was used previously and resulted in incomplete digestion.
High mock digestion (M_o) C_T values from most/all genes	
a) Insufficient DNA used in the digestion	Be sure to use at least the amount of DNA recommended in the protocol. Use the recommended methods and instruments to determine DNA concentrations. Be sure to include any RNase treatment steps recommended in the procedure of the chosen DNA isolation kit.
b) Degraded DNA	DNA samples may be contaminated by microbes due to improper storage of DNA samples, e.g., at 4°C. Always store DNA samples at –20°C (up to 2 years) or –80 °C (indefinitely).
c) PCR array or master mix incorrectly stored	Storing PCR array or master mix at inappropriate temperature for extended periods reduces their activity and PCR amplification efficiency.
d) Incorrect real-time PCR cycling program used	Be sure to use the correct cycling program, including 10 minutes at 95°C to fully activate the hot start enzyme in the RT^2 SYBR Green qPCR Mastermix.
All 4 digests (M_o, M_s, M_d, M_{sd}) C_T values for an individual gene are ≥32	
a) DNA sample may contain a different sequence relative to the most recent NCBI genome build	This may be due to unreported chromosomal abnormalities (insertion or deletions) or single nucleotide polymorphisms (SNPs) that affect the EpiTect Methyl II PCR Assays. Verification may require sequencing of the relevant genomic region in the original DNA sample.
b) Homozygous deletions	If the C_T values from all 4 digests for an individual gene, but not the majority of genes, are ≥32, genomic homozygous deletion most likely exists at this locus in the genomic DNA of the original sample.

References

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Cited references

1. Esteller, M. (2007) Epigenetic gene silencing in cancer: the DNA hypermethylome. Hum. Mol. Genet. **6**, R50.
2. Ordway, JM., et al. (2006) Comprehensive DNA methylation profiling in a human cancer genome identifies novel epigenetic targets. Carcinogenesis **27**, 2409.

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Product	Contents	Cat. no.
EpiTect Methyl II Signature PCR Array (22)	For methylation analysis of 22 genes in a 96-well or 384-well plate format 2, 12, or 24 x 96; 4 x 384	335212
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EpiTect Methyl II Custom PCR Array	For methylation analysis of customer-selected genes in a 96-well or 384-well plate format	335112
EpiTect Methyl II DNA Restriction Kit (12)	Reagents for the cleavage of methylated and unmethylated DNA for processing up to 12 DNA samples; 5x Restriction Digestion Buffer, Methylation-Sensitive Enzyme A, Methylation-Dependent Enzyme B	335452
EpiTect Methyl II PCR Assay (200)	Laboratory-tested forward and reverse primers for 200 x 25 µl reactions; 25 µl per primer; total volume: 200 µl	335002
DNeasy Blood and Tissue Kit (50)*	50 DNeasy Mini Spin Columns, Proteinase K, Buffers, Collection Tubes (2 ml)	69504
AllPrep DNA/RNA Mini Kit (50)	For 50 minipreps: AllPrep DNA Spin Columns, RNeasy® Mini Spin Columns, Collection Tubes, RNase-Free Water and Buffers	80204

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Product	Contents	Cat. no.
RT² SYBR Green qPCR Mastermix (2)*	For 2 x 96 assays in 96-well plates; suitable for use with real-time cyclers that do not require a reference dye, including: Bio-Rad models CFX96, CFX384, Bio-Rad/MJ Research Chromo4, Bio-Rad/MJ Research Opticon 2; Roche LightCycler 480 (96-well and 384-well); all other cyclers	330500
RT² SYBR Green ROX qPCR Mastermix (2)*	For 2 x 96 assays in 96-well plates; suitable for use with the following real-time cyclers: Applied Biosystems models 5700, 7000, 7300, 7500 [Standard and Fast], 7700, 7900HT 96-well block [Standard and Fast] and 384-well block, StepOnePlus; Eppendorf Mastercycler ep realplex models 2, 2S, 4, 4S; Stratagene models Mx3000P, Mx3005P, Mx4000	330520
RT² SYBR Green Fluor qPCR Mastermix (2)*	For 2 x 96 assays in 96-well plates; suitable for use with the following real-time cyclers: Bio-Rad models iCycler, iQ5, MyiQ, MyiQ2	330510
RT² PCR Array Loading Reservoir	5 ml capacity reservoir for convenient sample loading on PCR arrays	338162

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